European Journal of Molecular & Clinical Medicine

ISSN 2515-8260 Volume 10, Issue 03, 2023

Detection and comparison of different phenotypic methods of Biofilm formation in uropathogens and their correlation with antibiotic susceptibility pattern

[1]Dweep Jyoti Baishya (Assistant Professor),
[2]Rana Doley (Demonstrator),
[3]Atanu Chakravarty (Associate Professor),
[4]Bipanchi Mahanta (Assistant Professor),
[5]Geetumoni Sonowal (Assistant Professor),
[6]Monica Devi (PGT)

[*1,2,3,5,6] Department of Microbiology, Jorhat Medical College, Assam, India.
[*4] Department of Microbiology, Nagaon Medical College, Assam, India.

Address for Correspondence: Dr Dweep Jyoti Baishya.
Department of Microbiology, Jorhat Medical College, Jorhat- 785001
Assam, India.
E-mail: dbaishya78@gmail.com

ABSTRACT

Introduction: Urinary tract infection (UTI) is one of the leading causes of morbidity encountered in clinical practice. Emerging resistance of the uropathogens to the antimicrobial agents due to biofilm formation is a matter of concern while treating symptomatic UTI which leads to longer stay in hospital and increased cost of treatment. Detection of biofilm producer strains will guide the clinician in modifying antibiotic therapy for better clinical management and also help in designing adequate control measures as the isolates are also resistant to variety of disinfectants.

Aim: The present study was conducted to compare three methods Tissue Culture Plate (TCP) method, Tube method (TM) and Congo red agar (CRA) to detect biofilm formation by uropathogens and to correlate their susceptibility pattern with biofilm formation.

Materials and methods: A total of 150 culture positive significant isolates from urine samples were subjected to biofilm detection by TCP method, Tube method and CRA method. TCP was considered as gold standard method. Impact of biofilm production was correlated with the antibiotic resistant pattern.

Results: Out of 150 culture positive significant bacterial isolates, Gram negative organisms were isolated from 122 (81.33%) specimens and Gram positive growth was seen in 28 (18.67%) samples. *Escherichia coli* was the commonest Gram negative organism isolated (42.66%) while among Gram positive isolates, maximum biofilm production was shown by *Enterococcus faecalis* (66.66%). The gold standard TCP method detected 51 (34%) isolates as strong and 11(7.33%) isolates as moderate biofilm producers and remaining 88 (58.67%) isolates were weak/non-biofilm producing bacteria.

Conclusion: From our study we can conclude that TCP is the most reliable method for detection of biofilm formation in comparison to TM and CRA.
Keywords: Biofilm, Urinary tract Infection, Tissue culture plate, Tube method, Congo red agar

INRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infections. Around 95% of UTIs are caused by bacteria. Bacteria follow ascending route of infections in 90% of UTIs, primarily derived from fecal flora of the host, although hematogenous route of infections do occur. Emerging resistance of the uropathogens to the antimicrobial agents due to biofilm formation is a matter of concern while treating symptomatic UTI which leads to longer stay in hospital and increased cost of treatment.**[1]** Biofilms promote development of antimicrobial resistance by retarding diffusion of antimicrobials and facilitating plasmid exchange thus enabling dissemination of antimicrobial resistance.**[2,3]** Detection of biofilm producer strains will guide the clinician in modifying antibiotic therapy for better clinical management**[4]** and also help in designing adequate control measures as the isolates are also resistant to variety of disinfectants.**[3]** This emphasizes the need to screen all clinical isolates for biofilm production.

There are various methods to detect biofilm production like tissue culture plate (TCP), tube method (TM), Congo Red Agar method (CRA), modified CRA method (MCRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination**. [5]** The primary objective of the study is to compare three methods (i.e., TCP, TM and CRA) which can be used in routine clinical laboratory to detect biofilm formation by uropathogens

MATERIALS AND METHODS

Place and duration of the study

The study was conducted in Department of Microbiology, Jorhat Medical College, Jorhat, Assam from April 2022 to September 2022

Selection of isolates

Inclusion criteria: Uropathogens isolated from urine samples of all sexes of all age groups attending various outpatient departments and admitted in wards of Jorhat Medical College and Hospital were included in the study.

Exclusion criteria: Isolation of more than one bacterium, repeated isolates from the same patients who were on antibiotic therapy were excluded from the study.

A total of 150 mid-stream, clean catch urine samples from patients presenting with urinary tract infection were used for detection of biofilm forming bacteria.

Examination of urine

1. Macroscopic examination: Altered color, presence of turbidity, deposit noted.
2. Microscopic examination: Urine centrifuged at 2000 rpm for 15 min. The supernatant discarded, and wet preparation of sediment examined under low and high power to observe pus cells, red blood cell, cast and crystals, and epithelial cells.
3. Plating of the urine sample by standard loop technique: samples cultured by semi‑quantitative method on MacConkey's agar, Cystine Lactose Electrolyte Deficient (CLED) agar medium, and incubated at 37°C for 24 h. Urine culture yielding colony counts of $>10^5$ organisms/ml of a single type along with >10 pus cells/HPF of a centrifuged urine sample interpreted as diagnostic of bacteriuria. Bacterial counts of less than this considered insignificant and growth of more than

two types of organisms considered as contamination. The identification of the organism was performed by colony morphology, Gram staining, and standard biochemical tests.

4. Antimicrobial susceptibility testing was done using Muller Hinton agar by Kirby Bauer's disc diffusion method as per latest CLSI guidelines.

Reference strain of strong biofilm producer *S. aureus* ATCC 25923 and non biofilm producing strain *E. coli* ATCC 25922 were used as controls.

Ethical committee approval

Institutional ethical committee approval was obtained from Jorhat Medical College, Jorhat, Assam (Reference number – IEC (H) Reg. No. EC/NEW/INST/2020/1221.

Biofilm detection was done by the following methods:

Tissue Culture Plate method

Tissue Culture Plate (TCP) method as described by Christensen's *et al*., 1995 is the most widely used method and is considered as the gold standard method for detection of biofilm formation**.[6]** The isolates from fresh agar plates were inoculated in 5 ml of Trypticase soy broth and were kept for incubation at 37°C for 24 h. The cultures were diluted in 1:100 with fresh medium of Trypticase soy broth. Individual wells of 96 well–flat bottom polystyrene TCPs were filled with 0.2 ml aliquots of diluted culture. The uninoculated broth was added to the wells to check sterility and nonspecific binding of the media. The plates were then incubated at 37°C for 24 hours and after the incubation, contents were removed from plates by tapping gently. Plates were washed twice with 0.2 ml of phosphate buffer saline (pH 7.2) and incubated at 37°C for an hour. The plates were stained with 0.2 ml of 0.1% crystal violet for 10 min. Excess stain was removed by washing twice with deionized water and the plates were kept for drying. 200 µl of 33% glacial acetic acid was added to the wells. Optical density (OD) of the isolates was determined using micro ELISA auto reader (J MITRA ER181s) at a wavelength of 630 nm. The experiment was performed in triplicates. Biofilm formation was classified into Strong, moderate and weak/non-biofilm producers as shown in **Table 1**. The interpretation of biofilm production was done according to criteria of Stepanovic *et al*.**[7]** Optical density cut off (ODc) was calculated by the following formula:

Optical density cut off (ODc) = Mean optical density (OD) of negative control + 3X Standard Deviation (SD) of negative control

ODs of negative controls from reader-0.049, 0.057, 0.048, 0.054, 0.051, 0.059

Mean ODs of negative control= 0.053

1SD = 0.0044, 3SD= 0.0132

ODc= Mean + 3SD

= 0.053 + 0.0132

= 0.0662

Table 1: **Interpretation of biofilm production based on optical density values of Tissue Culture Plate method**:

Average OD value	Biofilm production
ODs≤ ODc (≤ 0.0662)	Negative
ODs< 2ODc (<0.13)	Weak
ODs 2X ODc ≤ 4XODc (0.13-0.264)	Moderate
ODs > 4ODc (≥ 0.264)	Strong

Tube Method

Christensen *et al*., 1995 described this qualitative method for the detection of biofilm**.[6]** 5 ml of Trypticase soy broth was inoculated with a loopful of organism and incubated for 24 hours at 37° C. Tubes were then decanted and washed with phosphate buffer saline (pH 7.2) and were allowed to air dry. Tubes were then stained with (0.1%) crystal violet for 10 min and washed with deionized water and left for air dry in inverted position. The scoring of biofilm formation was done based on the control strains used. The organisms were considered to be biofilm producers when there is formation of visible layer on walls and at the base of the tube while the formation of ring at the interference of the liquid medium indicated that the organism was non-biofilm producer. The experiment was performed in triplicates. The amount of biofilm formed was scored as (1) negative; (2) weak positive; (3) moderate positive; (4) strong positive.

Congo Red Agar method

Freeman *et al*., 1989 described a simple qualitative method to detect the biofilm formation by Congo Red Agar (CRA) method**.[8]** This method involves use of Brain Heart Infusion (BHI) agar with 5% Sucrose and Congo red in the following composition: BHI agar-52 g/L; sucrose-36 g/L; agar-10 g/L; congo red- 0.8 g/L. Congo red was prepared as concentrated solution and autoclaved. It is added to the medium when agar is cooled to 55°C and poured into petri plates. Plates were inoculated and incubated for 24–48 h at 37°C. Black colonies with dry crystalline morphology was considered positive for biofilm producing organisms while darkening of the colonies without dry crystalline colonial morphology indicates an intermediate result**.[9]** Non-biofilm producing organisms appeared to be pink in colour. The experiment was performed in triplicates.

Results

Out of 150 culture positive significant bacterial isolates from urine samples, Gram negative organisms were isolated from 122 (81.33%) specimens and Gram positive growth was seen in 28 (18.67%) samples. *Escherichia coli* was the commonest organism isolated (42.66%) followed by *Klebsiella pneumoniae* (16%). Among Gram positive organisms, *Enterococcus faecalis* was the predominant isolate (8%) followed by *Methicillin resistant Staphylococcus aureus* (6.67%) **(Table 2).**

Among the 150 bacterial isolates, 62 (41.33%) isolates showed biofilm formation by the gold standard Tissue culture plate (TCP) method. Maximum biofilm production was shown by *Enterococcus faecalis* (66.66%). *Escherichia coli* showed biofilm formation in 42.66% isolates followed by *Klebsiella pneumonia (*41.66%) and *Pseudomonas aeruginosa* (41.66%). **(Table 3)**

The gold standard TCP method detected 51 (34%) isolates as strong and 11(7.33%) isolates as moderate biofilm producers and remaining 88 (58.67% isolates were weak/non-biofilm producing bacteria **[Figure 1& 2, Table 4].** By Tube Method (TM), the number of organisms that showed strong biofilm formation was 23 (15.33%) and 49 (32.67%) organisms showed moderate and 78 (52%) isolates showed weak or no biofilm formation **[Figure 3, Table 4].** 48 (32%) and 28 (18.67%) isolates were strong and moderate biofilm producers by CRA method

respectively **[Figure 4 and Table 4]**. In our study we considered strong and moderate positive isolates as positive findings whereas weak positive and negative isolates were counted as negatives.

Statistical analysis was done for all the three methods to assess the sensitivity and specificity of these tests for detection of biofilm among bacterial isolates. TCP method was considered as the Gold standard for the study. **[5]** Comparative statistical analysis was done between TM and CRA with TCP. The parameters like sensitivity, specificity, positive predictive value and negative predictive value were calculated for both TM and CRA method. True positives were biofilm producers by TCP, TM and CRA methods **[Table 5].** False positive were biofilm producers by TM and CRA methods and not by TCP method. False negative were the isolates which were non biofilm producers by TM and CRA but were biofilm producing by TCP method. True negatives are those which were non biofilm producers by all the three methods. Sensitivity and specificity of CRA were 86.11% and 78.78% respectively, while the sensitivity and specificity of TM were 80.51 % and 83.87% respectively.

In our study, among biofilm producing Gram positive isolates Ciprofloxacin showed high percentage of resistance(86%). Erythromycin (56%), Cefipime (55%) and Clindamycin (53%) exhibited higher resistance pattern as compared to other antimicrobials used. **(Table 6)**

Among the biofilm producing Gram negative isolates, maximum resistance was seen to Amoxyclav (95%), Norfloxacin (90%), Ampicillin (90%), Ciprofloxacin (88%), Gentamicin (77%) and Aztreonam (66%) Minimum resistance was seen to Imipenem (9%), followed by Meropenem (12%) nitrofurantoin (22%), Piperacillin with tazobactam (41%), Amikacin (46%). **(Table 7)**

Table.2 Spectrum of organisms isolated

Organism	Isolates	Percent (%)
Escherichia coli	64	42.66%
Klebsiella pneumoniae	24	16%
Klebsiella oxytoca	6	4%
Enterococcus faecalis	12	8%
Enterococcus faecium	3	2%
Methicillin resistant Staphylococcus aureus	10	6.67%
Citrobacter species	3	2%
Acinetobacter baumannii	4	2.67%
Pseudomonas aeruginosa	12	8%
Proteus species	5	3.33%
Enterobacter species	4	2.67%
Methicillin sensitive Staphylococcus aureus	3	2%
Total	150	100%

Table.3 Organism wise distribution of biofilm production

Organism	Total Isolates	Biofilm producers	Percent (%)
Escherichia coli	64	27	42.18%
Klebsiella pneumoniae	24	10	41.66%
Klebsiella oxytoca	6	2	33.33%
Enterococcus faecalis	12	8	66.66%
Enterococcus faecium	3	1	33.33%
Methicillin resistant Staphylococcus aureus	10	4	40%
Citrobacter species	3	1	33.33%
Acinetobacter baumannii	4	2	50%
Pseudomonas aeruginosa	12	5	41.66%
Proteus species	5	2	40%
Enterobacter species	4	0	-
Methicillin sensitive Staphylococcus aureus	3	0	-
Total	150	62	

Table 4. Biofilm detection of 150 bacterial isolates by different phenotypic methods

Biofilm production	TCP, n(%)	TM, n(%)	CRA, n(%)
Strong positive	51 (34%)	23 (15.33%)	48 (32%)
Moderate positive	11(7.33%)	49 (32.67%)	28 (18.67%)
Weak positive	12 (8%)	23 (15.33%)	-
Negative	76 (50.67%)	55 (36.67%)	74 (49.33%)

Table 5: Comparison of Congo red agar (CRA) and Tube method (TM) with Tissue Culture Plate method (TCPM)

Screening Methods	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
CRA	86.11%	78.78%	81.57%	83.87%
TM	80.51%	83.87%	86.11%	77.61%

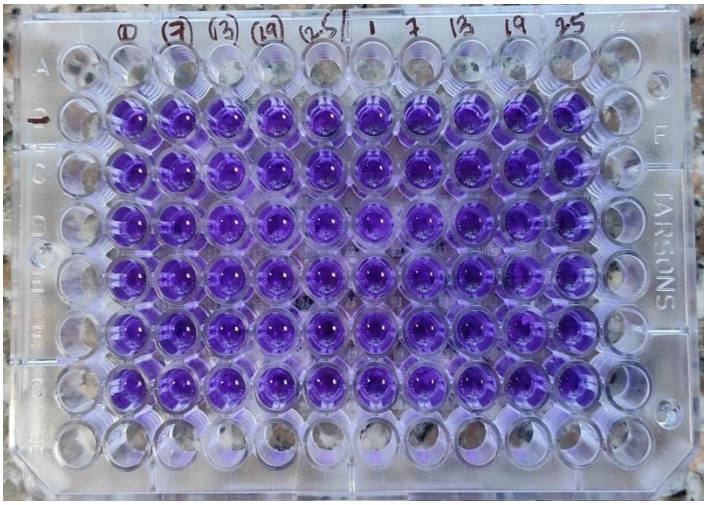
Table 6: Antibiotic resistance pattern of biofilm producing Gram positive isolates

Antimicrobial agent	Resistance pattern (%)
Ciprofloxacin	86%
Erythromycin	56%
Clindamycin	53%
Cotrimoxazole	46%
Gentamicin	43%

Cefipime	55%
Linezolid	12%
Nitrofuratoin	19%

Table 7: Antibiotic resistance pattern of biofilm producing Gram negative isolates

Antimicrobial agent	**Sensitive (%)**	**Resistance (%)**
Ampicillin	10	90
Cotrimoxazole	37	63
Ciprofloxacin	12	88
Norfloxacin	10	90
Gentamicin	23	77
Amoxycillin clavulanic acid	5	95
Amikacin	54	46
Piperacillin tazobactum	59	41
Imipenem	91	9
Nitrofurantoin	78	22
Aztreonam	34	66
Meropenem	88	12



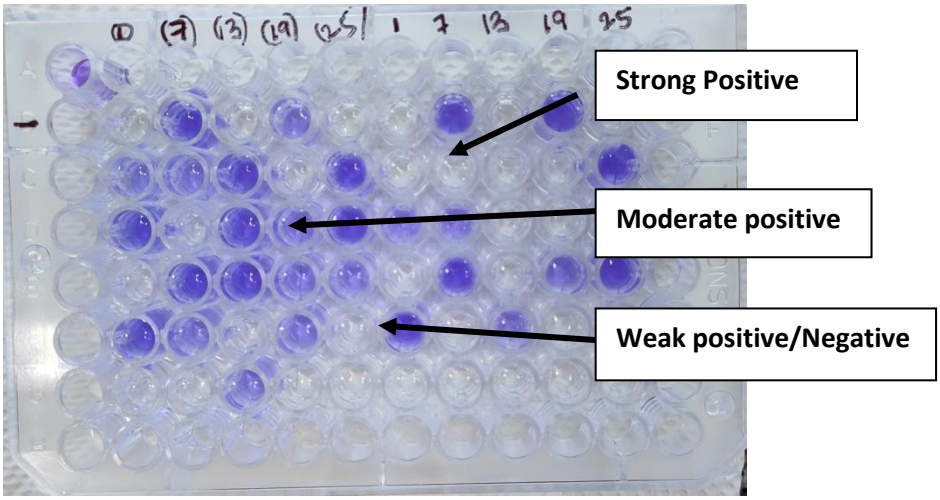


Figure 1 & 2: Screening of biofilm production by Tissue Culture Plate (TCP) method

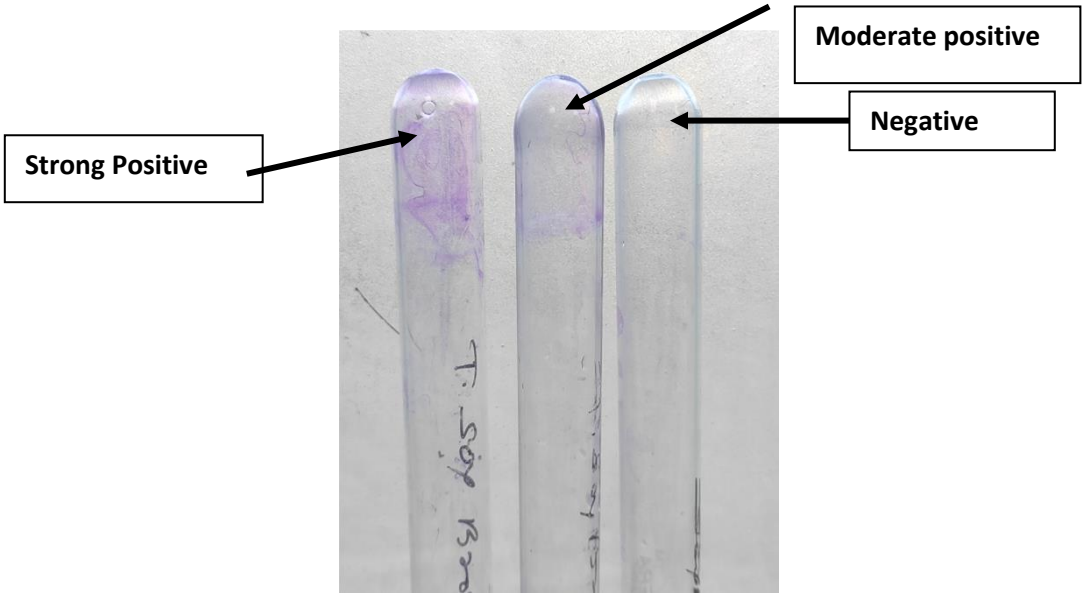


Figure 3: Tube method

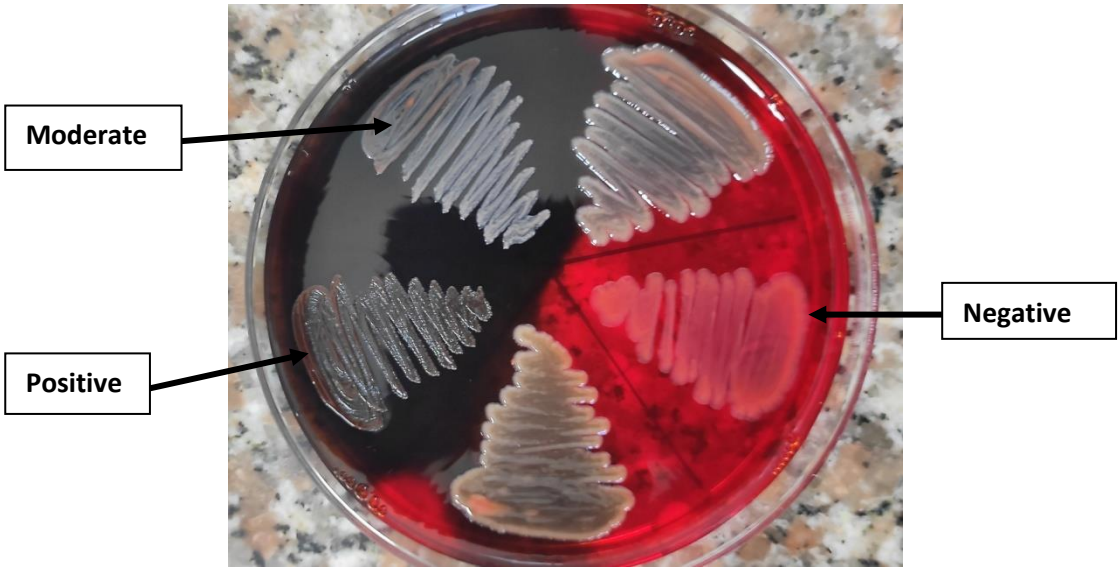


Figure 4: Congo red agar (CRA) plate method

Discussion:

UTI is considered as the most common bacterial infection worldwide causing significant morbidity and loss of work place productivity [**10, 11**]. UTIs have become a serious health threat with 250 millions of cases reported annually with much recurrence rate and chronicity **[12].** There is an increasing trend in the antimicrobial resistance among uropathogens and is attributed to formation of biofilms **[9, 13].** Biofilm producing pathogens play an important role in causing potentially fatal and persistent infections **[14].** Hence its detection should be mandatory in a laboratory set up **[15].** Biofilm detection can help the clinicians to formulate prompt effective therapeutic measures thereby help in reducing the morbidity and mortality associated with biofilm producing bacterial infections **[5, 16, 17].**

In the present study, Escherichia coli (42.66%) was the major isolate followed by Klebsiella pneumonia (16%). This is in accordance with studies by Kabir et al , Subramanium P et al, Jain et al and Tayal et al **[12, 18, 19, 10]** . Among Gram positive organisms, *Enterococcus faecalis* was the predominant isolate (8%) Noor *et al* , Jain *et al* and Tayal et al showed Enterococcus species to be the commonest Gram positive organism isolated in their studies (6% , 5.86% and 10.2% respectively) similar to the present study (8%) **[20, 19, 10]**

In the present study, majority of the biofilm producing isolates were E.coli (27%) followed by Klebsiella species (16%).These findings are in agreement with other studies conducted by, Niveditha et al, Shahidul KM et al and Mohammed EA et al. **[3,12,21].** However, Abdagire et al **[22]** found higher biofilm production by *S. aureus* (60.15%) followed by *E. coli* (39.58%).

In our study biofilm was detected in 41.33 % of the urinary isolates by TCP method whereas TM and CRA method showed 48% and 50.66% respectively. This is in concordance with the study by Saroj et al **[23]** Tayal et al **[10],** Samidurai et al **[17]** and T A Dhanalakshmi et al **[24].** However Bose et al **[16]** and Pallavi et al **[25]** detected only 6.15% and 9.73% respectively by CRA method. **(Table 4)**

In the present study, sensitivity and specificity of CRA method was found to be 86.11% and 78.78% respectively. This in concordance with studies by Adilson et al **[26],** Tayal et al **[10]** and T A Dhanalakshmi et al **[24].** However, other studies by Mathur et al **[27],** Bose et al **[16]** and Chandana et al **[13]** found very low sensitivity for CRA method. **(Table 9)**

The sensitivity and specificity of TM was found to be 80.51% and 83.87% respectively. This is almost similar to other studies by Mathur et al **[27],** Tayal et al **[10]** and Chandana et al **[13].** Observer differences were noted while differentiating weak biofilm producers from non-biofilm producers. Mathur T et al., **[27]** and Tayal et al., **[10]** also reported subjective errors in tube method. **[Table 9]** shows the statistical evaluation of biofilm detection methods in different studies. The variations observed in various studies might be because of the differences in the sources from which the strains were isolated and differences in the methodology employed in the study.

In our study, among biofilm producing Gram positive isolates Ciprofloxacin showed high percentage of resistance(86%). Erythromycin (56%), Cefipime (55%) and Clindamycin (53%) exhibited higher resistance pattern as compared to other antimicrobials used. Among the biofilm producing Gram negative isolates, maximum resistance was seen with Amoxyclav (95%), Norfloxacin (90%), Ampicillin (90%), Ciprofloxacin (88%).

In a study conducted by Poovendran *et al.*, **[29]** all biofilm forming strains were maximum resistance to amoxyclav (100%), followed by chloramphenicol (100%), gentamicin and cefotaxime (86% each), ceftazidime (84%), cotrimoxazole, and piperacillin with tazobactam (83% each), and amikacin (70%). The study conducted by Sevanan *et al.* **[30]** showed that biofilm producing organisms are more resistant to antibiotics compared to nonbiofilm producing isolates. The resistant pattern of erythromycin, amikacin, co‑trimoxazole, ampicillin, meropenem, chloramphenicol, tobramycin, and gentamicin were found to be in the order of 90.6%, 71.9%, 65.6%, 59.3%, 56.3%, 56.3%, 53.1%, and 50.0%, respectively among biofilm producing isolates.

Table 8: Biofilm detection by different methods observed in various studies

Authors (year)	Tissue culture plate method (%)	Tube method (%)	Congo Red agar method (%)
Bose et al **[16]** (2009)	54.19	42.46	6.15
Saroj et al **[28]**	56	48	72

(2012)			
Tayal et al **[10]** (2015)	27	37.96	40.88
Pallavi et al **[25]** (2017)	69.91	53.09	9.73
Samidurai et al **[17]** (2017)	45.71	42.86	42.86
T A Dhanalakshmi et al **[24]** (2018)	39.77	38.26	46.97
Present study	41.33	48	50.67

Table 9: Statistical evaluation of Tube and Congo red agar method in various studies

Authors (years)	Tube method				Congo red agar method			
	Sensitivity (%)	Specificity (%)	PPV* (%)	NPV** (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Mathur et al **[27]** (2006)	73.6	92.6	93.4	66.6	6.8	90.2	66.6	25.3
Bose et al **[16]** (2009)	76.27	97.56	97.36	77.66	8.25	96.34	72.72	47.02
Adilson et al **[26]** (2010)	100	100	-	-	89	100	-	-
Hassan et al **[5]** (2011)	73	92.5	94	66	11	92	73	37
Tayal et al **[10]** (2015)	94.59	83	-	-	94.59	81	-	-
Chandana et al **[13]** (2015)	71.8	88.8	-	-	12.7	86.2	-	-
Pragyan et al **[9]** (2016)	81	95.1	93.3	85.6	16.8	93.9	67.9	57.3
Pallavi et al **[25]** (2017)	74.70	96.85	97.87	65.42	11.24	98.43	93.33	36.13
T A Dhanalakshmi et al **[24]** (2018)	63.81	78.62	66.34	76.69	80	75.47	68.29	85.11
Present Study	80.51%	83.87%	86.11 %	77.61 %	86.11%	78.78%	81.57 %	83.87 %

Conclusion: Biofilms are a major cause of recurrent and recalcitrant urinary tract infection (UTI), leading to increased morbidity in the patient, increased duration of hospital stay and increased economic burden. The formation of biofilm might also be the reason for the emerging resistance of antimicrobial agents in patients with urinary tract infection. We can conclude from

our study that TCP is a quantitative and reliable method to detect biofilm forming microorganisms. When compared to TM and CRA methods, and TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

Acknowledgement: We are thankful to all the staff and technicians of Department of Microbiology, Jorhat Medical College for their support and cooperation

Conflicts of interest
There are no conflicts of interest.

Financial support: None

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